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ABSTRACT

The current standard operating procedures for the Salmonella/microsome (S/M) plate incorporation assay are described. Methods for reisolation of tester strains TA98 and TA100 are outlined, as well as procedures for maintenance of working cultures in frozen permanent and frozen ampoule cultures. The procedure for analyzing and making transitions to new batches of Aroclor® 1254-induced rat liver microsomes is presented. Recipes and additional procedures for preparing all of the necessary solutions, media, etc. are given, as well as lists of equipment, chemicals and suppliers.

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LIST OF ABBREVIATIONS

2AA	2-aminoanthracene
Abs	absorbance
Bio	Biotin
^{o}C	degrees centigrade
CV	crystal violet
CSC	cigarette smoke condensate
DMSO	dimethyl sulfoxide
^{o}F	degrees Fahrenheit
FW	formula weight
g	gram
G-6-P	glucose-6-phosphate
His	histidine
IT	impaction trapped
M	molar
mg	milligram
ml	milliliter
mM	millimolar
NA	nutrient agar
NaP	sodium phosphate
NADP	nicotinamide adenine dinucleotide phosphate
NaN_3	sodium azide
2NF	2-nitrofluorene
nm	nanometer
O.D.	optical density
ON	overnight
QS	as much as suffices (amount) to bring to a designated volume
R-factor	gene for ampicillin resistance
rfa	deep rough mutation

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2R1	University of Kentucky blended reference cigarette
S/M	Salmonella/microsome
S9	9000 x g rat liver supernatant
+S9	with metabolic activation (+S9 mix)
-S9	without metabolic activation (-S9 mix)
TPM	total particulate material
TPN	triphosphopyridine nucleotide; NADP
u	micron
ug	microgram
uvrb	ultra-violet repair B gene
VBMG	Vogel Bonner Minimal Glucose

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I. INTRODUCTION - BRIEF HISTORICAL PERSPECTIVE

The Salmonella/microsome (S/M) plate incorporation assay (Ames test) was developed by Bruce N. Ames and coworkers at the University of California, Berkeley, CA, to detect potential carcinogens and mutagens [1,2]. Several histidine requiring (deficient) bacterial tester strains of Salmonella typhimurium were engineered to detect (by reversion) chemical induced damage to DNA (e.g., frameshift mutations, base pair substitutions). Metabolic activation or conversion of carcinogens to their active mutagenic forms (simulating mammalian systems) was achieved with the addition of rat liver homogenates to the assay mixture. This assay was thoroughly validated in Ames' laboratory using 300 chemicals representing carcinogens and non-carcinogens of a wide variety of chemical types [16, 17], and the assay was further improved by the introduction of more sensitive strains (e.g., TA98 and TA100) [18]. With the introduction of additional strains [32,33,46] and changes in methodology, revised assay procedures were recently published [14]. The popularity of this assay in short term testing batteries has been realized by its widespread use worldwide and the proliferation of test data on more than 5000 chemicals [47,48].

Various tester strains (TA1530, TA1535, TA1537 and TA1538) were first obtained from either Dr. R. W. McCuen (when he came to Philip Morris from Sloan Kettering), Dr. H. Rosenkranz, and/or Dr. B. N. Ames. Of these strains, TA1538 was established as the strain of choice. Using the Rosenkranz protocol [49], Dr. W. K. Neal, J. J. Booker, and M. F. Minor established the Salmonella/microsome (S/M) assay at Philip Morris with positive control compounds and Elmenhorst trapped cigarette smoke condensate from 1R1 and X6DOAD cigarette models [6,7]. The assay was then validated with ten model CSCs [8], and newly engineered tester strains, TA98 and TA100, were substituted as the strains of choice for testing because of their increased sensitivity to tobacco smoke products (particularly TA98) over their respective parent strains (TA1538 and TA1535). A changeover to the widely used Ames protocol [2] was also effected at this time. As the methodology and use of the assay evolved, impaction trapped CSCs (see reference 13 for IT CSC preparation), fractions [3,9], pyrolyzates [5],

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sidestream smoke [10], TPM [44, 45], potential cigarette additives [35], etc. were tested. Studies were conducted and protocols established to make transitions between batches of Aroclor® 1254-induced rat liver microsomes (S9) [19,20,21,29,30]. Comparisons of rat S9 versus hamster S9 using positive control compounds and 2R1 IT CSC were also evaluated [22]. The incorporation of methods to evaluate toxicity in the assay were studied and documented [42]. In short, the S/M assay has been established as a short term bioassay which is being used extensively at PM to study/determine the biological activity of tobacco smoke products and potential ingredients. (Note: The author refers the reader to Project 6906 Annual Reports from 1974 to 1987 [55-68] for a more comprehensive survey of test samples, research programs and references.)

The assay methods which are currently being used were revised following the participation of PM in the ICI genetic drift study [28,34], in addition to input gained directly from Ames' laboratory [26,31]. These changes have been extensively outlined in these references and parallel some of the revisions suggested by workers in Ames' laboratory [14]. The rest of this report will document the standard operating procedures that were adopted and are currently being used routinely by Project 6906 personnel.

STANDARD OPERATING PROCEDURES

II. Safety Precautions

The safety of the personnel working with biologically active samples is of primary concern. The work areas for this work have been designated. The weighing and makeup of hazardous chemicals are performed in a glove box, and a special storage facility for these chemicals has been established [50, 51]. The procedure for weighing hazardous materials has been thoroughly documented in reference 51. Dilutions of positive control compounds for testing are made in a chemical fume hood lined with absorbant plastic backed paper matting. The assays are performed on laboratory tables lined with the same protective matting. Personnel are required to wear disposable lab coats and disposable gloves (plastic or latex depending on the solvents used in the assay). Contaminated gloves and other articles are discarded in

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designated hazardous waste containers and are replaced immediately during the conduct of the assay.

The procedures for the handling and disposal of solid and liquid hazardous wastes have been previously documented [52-54]. The protocol for the solid waste disposal procedures has been revised: a) the storage area for empty/full drums is now located in a designated area of the R&D stockroom facility; and b) Project personnel are responsible for the transport of these barrels to and from the stockroom facility.

All work areas are routinely swabbed with 70% or 95% ethanol solutions. Even though the strains are not pathogenic [2], precautions are still taken to autoclave unused cultures and nondisposable glassware in contact with cultures for 30-45 minutes at 250°C before washing this glassware. Wherever possible, disposable materials are used (e.g., petri plates, test tubes, etc.).

III. TA98 AND TA100 CULTURES

A. Culture Maintenance [27,24,28]

1. Store strains at -80°C as either frozen permanents or frozen ampoule cultures:
   a. Use the frozen permanents to make up large batches of frozen ampoule cultures.
   b. Use the frozen ampoule cultures to inoculate overnight (16 hour) culture broth media.

2. Reisolation (see below) of a strain is necessary if there is:
   a. Loss in cell viability;
   b. Dramatic increase or decrease in spontaneous revertants; or
   c. Loss of a marker trait (e.g., ampicillin resistance, uv sensitivity, crystal violet sensitivity).

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B. Growth of Overnight Cultures [14,23,31]

1. Thaw a frozen ampoule of the appropriate culture(s).

2. Inoculate 100 ml of sterile Oxoid nutrient broth supplemented with 10 ug/ml L-histidine HCl (room temperature) with 0.2 ml of the thawed frozen ampoule culture. For a large experiment, inoculate enough flasks to give excess culture media for the experiment.

3. Cap culture flask(s) loosely and tape if necessary to keep in position.

4. Place flask(s) in a 37°C orbital shaker bath and incubate at 150 rpm overnight for 16 hours.

5. After 16 hours, remove cultures from the bath and store either on ice or refrigerated. If more than one culture flask was required, combine cultures in a sterile screw capped flask. Only fresh cultures are used to conduct the assay.

C. Optical Density Measurements

The optical density of each overnight culture is now routinely measured. These data have been used to ensure that the cell concentration of the culture is within the suggested range for use in an assay [2, 14, 34]. A measurement that is either too high or too low is an immediate indicator that there may be a problem with the culture before an experimental assay has been conducted. Initially, the optical density measurements were derived from a procedure using a Klett-Summerson photoelectric colorimeter [34]. Because of intermittent problems with the Klett system, a more reproducible procedure has been developed using a Hitachi Model

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150-20 spectrophotometer [40]. A brief outline of the automated procedure is described below.

1. Turn on the spectrophotometer and sample sipper.
2. Set the peristaltic pump and release clamp.
3. Enter the date and time.
4. Select option #1 (Photometry) and enter.
5. Press the memory recall parameter list display and select memory program #6 (Absorbance) and enter.

   Note: If the Absorbance program has been deleted, repeat step 4 above and enter the following:

Wavelength	650 nm
Mode	Absorbance
Response	Medium
Remark	Cell Concentration vs Absorbance

6. Set up the reference blank: Using a clean disposable cuvette (wipe sides with a Kimwipe), fill with 5 ml of sterile Oxoid nutrient broth culture media supplemented with 10 ug/ml L-hisitidine HCl. Place blank in the cuvette holder with the clear side facing the light source.
7. Wash the sample sipper: Pour distilled water into a flask, insert aspiration tube into the flask and press the wash key several times.
8. Repeat step 7 with the sterile nutrient broth culture media and auto zero the instrument.
9. Measure the optical density of overnight cultures in the same way by a) inserting the aspiration tube into the culture sample (usually about 5 ml); b) pressing the sipper lever once; and c) waiting for the result to print out on the data processor. An acceptable absorbance range has been determined for tester strain TA98 to be between 1.40 and 1.65 [40]. An acceptable range is currently not available for TA100.
10. Shut down the instrument in the following order:
    a. Release the peristaltic pump and close the clamp;
    b. Turn off the sample sipper, followed by the spectrophotometer.

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D. **Serial Titer Dilutions**

Serial titer dilutions are made with each overnight culture prior to assay to determine the viable counts.

1. For each series of dilutions, prepare six sterile culture tubes by dispensing 4.5 ml of saline (see solution 6) into each tube.
2. Serially dilute the overnight (16 hour culture) to be used for testing as follows:

tube	ml saline	ml culture	~ cells/.1 ml
#1	4.5	0.5 ml of the straight 16 hour culture	10 E07
#2	4.5	0.5 ml of dilution tube #1	10 E06
#3	4.5	0.5 ml of dilution tube #2	10 E05
#4	4.5	0.5 ml of dilution tube #3	10 E04
#5	4.5	0.5 ml of dilution tube #4	10 E03
#6	4.5	0.5 ml of dilution tube #5	10 E02

3. Plate 0.1 ml of the last dilution set in triplicate on VBMG agar test plates (± ampicillin) in 2.5 ml soft agar with excess histidine present to provide titers or viable counts.

4. For the actual assay, dispense 0.1 ml of the straight overnight (16 hour) culture per assay tube with a sterile 0.5 ml repipet.

E. **Strain Reisolation, Frozen Permanents and Frozen Ampoule Cultures [27,24,28,14]**

1. **Single Colony Isolates**: Obtain single colony isolates of each strain from frozen permanent or frozen ampoule stock cultures on hand, or from discs or agar slants provided by Ames' laboratory.

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a. Using a sterile loop, streak culture across an Oxoid nutrient agar plate supplemented with L-histidine HCl (10 ug/ml) and ampicillin (~25 ug/ml).

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b. Cross streak the surface of each plate using a sterile loop, followed by streaking and cross streaking a number (2-3) of additional Oxoid nutrient agar plates (+ histidine; + ampicillin) to achieve a serial dilution of the inoculum. These operations are ideally performed in a horizontal laminar flow hood.

c. Place plates (inverted) in a 37°C incubator and incubate for 48 hours.

d. Pick four or five single colony isolates from the surface of the plate with a sterile loop and inoculate separate 100 ml aliquots of Oxoid nutrient broth supplemented with 10 ug/ml L-histidine HCl and ~25 ug/ml ampicillin.

e. Incubate these broth cultures, each representing a single colony isolate, at 37° C for 16 hours at 150 rpm in a shaking water bath.

2. **Preparation of frozen permanents:**

a. Mix 5 ml of each overnight (16 hour) culture with 0.4 ml DMSO.

b. Dispense 1 ml aliquots of each mixture into Wheaton cryules and store frozen at -80°C. These will be referred to as the frozen permanent cultures.

c. With the culture media remaining after making the frozen permanents, check each culture for the spontaneous mutation, viability of plates with and without ampicillin, and for crystal violet, ampicillin disc and uv sensitivity.

Marker Checks:

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1. To check ampicillin resistance (R-factor) and crystal violet sensitivity (deep rough mutation; rfa), plate 0.1 ml of the overnight culture, 0.1 ml of Super His (50.42 ug/ml L-hisitidine HCl), and 2.5 ml soft agar supplemented with 0.05 mM biotin and 0.05 mM histidine HCl. Allow soft agar to harden and then place ampicillin discs and BBL

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discs with 10 ul of a 1 mg/ml crystal violet solution on their respective test plates. A good culture will show no response around the ampicillin disc and a measurable zone around the crystal violet disc (see Table 1).

2. The purpose of the uv sensitivity test is to confirm the uvrB mutation. This is accomplished by streaking the 16 hour, overnight cultures on Oxoid nutrient agar plates with a sterile wire loop. Next, cover half of each test petri plate with foil (or other suitable material). Irradiate the plate with uv light at a distance of 33 cm for 8 seconds. Incubate at 37°C for 12-24 hours. The side of the petri plate exposed to the uv light should not show any viability.

d. Also, conduct a small S/M experiment with each culture to determine each culture's response to the appropriate positive control compounds and to 2R1 IT CSC.

e. Chose the best frozen permanent culture for each strain based on the data obtained above and the criteria specified for each strain [14]. See Table 1 for the acceptable values for TA98 and TA100. Discard all of the other frozen permanent cultures.

f. Use these frozen permanents to make a large batch of overnight culture from which ~ 70 frozen ampoule working cultures can be made.

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Table 1. Acceptability Criteria for TA98 and TA100 Cultures

	TA98*	TA100**
Klett Reading	140-180	145-205
Optical Density	1.40-1.65***	****
uv Exposure	total kill	total kill
Crystal Violet Disc (mm zone)	14-18	14-18
Ampicillin Disc	no zone	no zone
Average Titer ± Ampicillin (0.1 ml of 10 -E06 dilution)	100-200	120-200
Average Spontaneous Reversion (O&C)	15-60	120-210
Average Spontaneous Reversion (DMSO)	15-60	120-200
2R1 CSC (revertants/mg CSC)	1200-2800	500-1200

* Reference 24.

** Reference 70.

*** Reference 40.

**** Data not currently available.

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3. **Preparation of frozen ampoule working cultures:**

a. For each strain, thaw a frozen permanent.

b. Inoculate 100 ml of Oxoid nutrient broth No. 2 fortified with 10 ug/ml L-histidine HCl and ~ 25 ug/ml ampicillin with 0.2 ml of the frozen permanent culture.

c. Incubate overnight (16 hours) at 37°C in an orbital shaking water bath (150 rpm).

d. Measure the optical density of each overnight culture.

e. Add 7.4 ml DMSO to 92 ml of each overnight culture and dispense 1 ml aliquots in to Wheaton cryules (2 ml) using a sterile 1 ml repipet dispenser. Filling the cryules at least one-half full helps prevent oxidative damage to the culture [26].

f. Store ampoules in trays at -80°C.

g. Make up fresh overnight cultures of TA98 and TA100 using 0.2 ml from these frozen ampoules as inoculum into 100 ml Oxoid nutrient broth fortified with 10 ug/ml L-histidine HCl (no ampicillin) at 37°C and 150 rpm.

h. Check these cultures for their spontaneous mutations, the appropriate markers, and for their response to positive control compounds and 2R1 CSC [28,24,14]. The acceptable responses for TA98 and TA100 are given in Table 1.

Note: If reisolation of the culture is not necessary, but more frozen ampoule working cultures are needed for routine use, ampoules may be prepared from an existing frozen permanent by simply following the steps listed above.

IV. RAT LIVER MICROSOMES (S9)

The transition from one Aroclor® 1254-induced rat liver microsome preparation (S9) to another periodically occurs [19,20,21,29]. Initially, the S9 was prepared at the University of Richmond by PM personnel (W. K. Neal; J. J. Booker; K. E. McCarty). When Litton Bionetics became a

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commercial source of S9, several batches were purchased but problems with contamination that could be traced to the S9 were sporadic. Currently, S9 preparations are being purchased through Litron Laboratories [30].

A. Preparation of S9 Fraction:

The protocol for making Aroclor® 1254-induced rat liver microsomes is thoroughly presented in the revised methods paper [14]. These 9000 x g supernatants are being prepared by Dr. A. Tometsko according to this protocol (Litron Laboratories).

B. S9 Evaluation:

Routinely, experiments are carried out to compare an old batch of S9 to a new batch prior to making a transition to the new S9. The experimental design used to compare microsome preparations has been documented [29], as well as recommendations made by J. Tindall to improve these evaluations. It was evident that each microsomal preparation did not assay identically in terms of the relative activities of model IT CSCs when making the transition from one microsome preparation to another [29]. Even though the statistical analyses of the data showed significant differences between the S9 preparations, the overall rank order of responses for the Model I CSCs was not different. As long as the readout or conclusion (the ranking of the Model I CSCs) remained unchanged, regardless of the protein level or the microsome preparation used, other differences in the preparations were considered to be insignificant. The S9 evaluations are conducted as follows.

1. Determine the protein content according to the modified Lowry method [29, 41].
2. Design the S9 mix to test the S9 at two levels: 35 ul/plate and 45 ul/plate (see + S9 mix makeups in Appendix A).
3. IT CSC: Prepare two smokings of the ten Model I cigarettes according to standard procedures [13]. Test two doses of each IT CSC at 0.1 and 0.2 mg/plate.
4. To evaluate the resulting data, perform statistical analyses of variance in order to compare the data and determine the differences in the slope (specific activity) data [29].

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V. PLATE INCORPORATION ASSAY:

A. Sample Request

Many of the samples (CSCs, potential tobacco ingredients, fractions, etc.) tested in the Salmonella/microsome assay are generated and submitted for testing by personnel outside of Project 6906. To accomodate these requests, a standard request form has been devised to define the objective of the request and to gather enough information and background about the sample(s) so that Project 6906 personnel are capable of understanding and facilitating the testing properly. The information requested is very straightforward, and a sample form is shown in Table 2. This form also allows the tracking of the request from the time it is received until the time when results are released. Generally, an appointed sample coordinator (the project leader or project member) is assigned the responsibility of keeping these tracking records and determining the order of priority for testing samples.

B. Experimental Design

Based on the information supplied on the sample request form (Table 2), the experimenter should be able to adequately define the experimental objective and to design the overall experiment. Table 3 represents the form used to help formulate the experimental design. Once this is filled out completely, the experimenter can determine the number and kinds of plates [e.g., nutrient agar plates, Vogel Bonner minimal glucose (VBMG) agar plates, etc], the culture (type and amount), the enzyme (record S9 batch, amount of S9, amount of S9 mixture needed), and the amount of -S9 mix needed. The sample codes and doses which are also recorded on this form help the experimenter set up sample and dose sets for making computer labels [11]. Generally, samples (test and control) are tested in triplicate. The same is true for titers plated with and without ampicillin incorporation in VBMG agar plates. The ampicillin disc, crystal violet, and soft agar

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Table 2. Salmonella/Microsome Assay Request Form

SALMONELLA/MICROSOME ASSAY

REQUEST FORM

Submitter _____

Date of Request _____

Purpose _____

For 6906 Use Only
Date request rec'd _____
Date samples rec'd _____
Date samples tested _____
Tester Strain(s) _____ (ampoule) _____
_____ (ampoule) _____
S9: Aroclor _____; _____ µl of S9/plate
Experimenter(s) _____
Notebook/page Nos. _____
Date results released _____

Total Number of Samples _____ Stock Conc. (mg/ml) _____ Vol. (ml) _____
Recommended Solvent _____ Test Doses (mg/plate) _____
Experimental Parameters (Circle appropriate choice):

1. Tester Strain(s) ? TA98 TA100 Other _____
2. Activation ? -S9 +S9 ± S9
3. Procedure ? Ames Micro

Sample Name or Code	Notebook Ref. No.	Sample Description, and Other Important Sample Information or History	Delivery mg/cigt.	Comments

Smoking Notebook Ref. No. _____

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Table 3. Salmonella/Microsome Assay Sample Data Sheet

Purpose:

Method _____
Enzyme _____
Culture _____
Experiment _____
Date _____

	TA98	TA100
Titer		
AMP		
C.V.		
O + C		

Overlay	VB	NA
O + DMSO		
O + (-S9)		
O + (+S9)		
O + S9 Fr.		
O + Sup-His		

SAMPLE CODE	DOSE(S)	mg ml	TA98		TA100	
			-M	+M	-M	+M

overlay plus cells (O&C) markers or controls for each test culture are plated with 2.5 ml of the soft agar and 0.1 ml of the overnight culture on VBMG plates. For titers (± ampicillin), CV and ampicillin disc marker checks, 0.1 ml of a sterilized 50.42 ug/ml histidine HCl solution (Super His solution) must be added prior to plating the ON culture (or dilution) on VBMG agar plates. The form in Table 4 is helpful for calculating the amount of soft agar, +S9 mix, and -S9 mix required for each assay. The amounts of the +S9 mix components may be double checked using the 35 ul/ml S9 mix table in Appendix A. The makeup and routine dilution schemes for the positive controls and CSCs are also given in Table 4. The procedure for preparing the standard test dilutions of potential tobacco ingredients is also presented in Appendix A. The solvents most widely used are DMSO and water. For reference, other solvents compatible with the assay have been published [15].

C. Bar Code Labels and Random Testing Order of Samples

Bar code labels are routinely generated for all of the assay plates and for the application to the caps of the sterile sample (positive controls and test samples) dilution tubes according to procedures previously documented [11]. To reduce the possibilities of systematic errors or variation occuring during the experiment, random testing orders of the experimental samples may be generated as an option. When a random running order is specified, a random order sheet is automatically printed out as a record for the notebook. Additionally, a computer printout of all the other experimental parameters and initialization data is generated for notebook or file records.

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Table 4. Salmonella/Microsome Assay Preparation of Materials Sheet

Notebook:_____ Experiment:_____ Date:_____

"SOFT AGAR" _____plates X 2.5ml/plate = _____ml needed, Make _____mls

.6g agar
.5g NaCl / 100ml D·D H_2O X___ = _____g agar ___#_____ / _____ml D·D H_2O
+
10ml His-Bio Soln. X___ = _____ml His-Bio Soln. _____

S9 MIX _____plates X 0.5ml/plate = _____ml needed,make _____ml

	per 1.0 ml		per _____ml	Stock Ref.
Salts	0.02ml		_____ml	
NaP Buffer	0.50ml	0.93 X_____ =	_____ml = ___ml	_____
D·D H_2O	0.41ml		_____ml	_____
G-6-P	0.001531		_____g	_____
TPN	0.003434g		_____g	_____
S9 Fraction	0.07		_____ml	_____
	1.0 ml		_____ml	

-S9 MIX _____ "-S9" plates X 0.5ml/plate = _____ml needed, make _____ml

POSITIVE CONTROLS

2AA (2-aminoanthracene)--for TA98 and TA100 (+S9), use____ug/plate/100ul

2AA: 200ug/.1ml (A) → 0.1ml(A) 1.9ml DMSO → 100ug/ml (B) → 0.1ml(B) 1.9ml DMSO → 5ug/ml (C)

2NF (2-nitrofluorene)--for TA98 (-S9), use_____ug/plate/100ul

2NF: 50ug/.1ml (A) → 0.1ml (A) 1.9ml DMSO → 25ug/ml (B) → 25ug/ml (B)

NaN_3 (Sodium Azide)--for TA100 (-S9), use_____ug/plate/100ul

NaN_3: 200ug/.1ml (A) → 0.1ml (A) 1.9ml H_2O → 100ug/ml (B) → 0.1ml (B) 1.9ml H_2O → 5ug/ml (C)

2R1 CSC (Kentucky Reference)--for TA98 and TA100 (+S9), use 100ul/plate

2R1 stock = 10mg CSC/ml DMSO (Doses: 0.05, 0.1,0.15, and 0.2 mg/plate)

MG/PLATE	MG/ML STOCK	ML DMSO
0.05	0.050	0.950
0.10	0.100	0.900
0.15	0.150	0.850
0.20	0.200	0.800

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D. Experimental Preparation Checklist

Each assay requires a tremendous amount of preparation. The following is a list of what can be done in advance, as well as the duties that are necessary to perform the day prior to and the day of the experiment. These tasks are generally shared and coordinated between two laboratory personnel.

1. **Advance Preparation:**
   a. Inventory and make all of the necessary solutions [See Appendix A].
   b. Pour agar plates [See Appendix A]: 30 ml of agar media per plate.
   c. Sterilize glassware and equipment: culture bottles, flasks, repipets and plungers, agar flask, test tubes (with and without disposable caps), stir bars.
   d. Labels:
      1. Generate labels.
      2. Number labels in succession according to the running order of the experiment.
      3. Apply labels to the plates and tubes.
   e. Label bottles or flasks to be used.
   f. Set up the assay station: circulating bath for soft agar, stir plates, lead donuts, container to hold +S9 mix and cultures on ice, vortexer, pipetting devices, sterile tips, and waste bag.
2. **Preparation the Day Before the Assay:**
   a. See list of advance preparation above.
   b. Inoculate nutrient broth and incubate for 16 hours at 37°C and ~150 rpm (see page 4 for the procedure).
   c. Set up dilution tubes for titers (dispense 4.5 ml of sterile saline into capped, sterile titer tubes and refrigerate), -S9 tubes, and tubes for plating requiring excess histidine (add 0.1 ml Super His solution to sterile tubes for plating titers, crystal violet and ampicillin discs).
   d. Weigh out ingredients for soft agar.

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e. Weigh out cofactors [glucose-6-phosphate (G-6-P) and NADP (nicotinamide adenine dinucleotide phosphate; TPN)] and store in a freezer at -20°C.

f. Premeasure the amount of the regular histidine-biotin soft agar supplement needed.

3. **Preparation the Day of the Experiment:**

a. Cultures:

1. Check optical density of the overnight culture.
2. Make the titer dilutions.
3. Refrigerate or keep culture on ice prior to the assay.

b. Turn on autoclave, circulating water bath (for soft agar), and standing water bath.

c. Soft Agar: Make up the requisite amount of soft agar and sterilize by autoclaving. Cool to approximately 45°C in a standing water bath. Add 10 ml of 0.5 mM biotin + 0.5 mM L-histidine HCl per 100 ml of soft agar. Mix well. Pour supplemented soft agar into the agar flask and place in a beaker of 45°C water heated by a circulating water bath coil.

d. Thaw CSC stock solutions in a 37°C incubator.

e. Thaw positive control (2AA or 2NF) stock solutions just prior to the assay time.

f. Make dilutions of positive controls, CSCs, and/or potential cigarette ingredients (dilutions of the positive controls are performed in a chemical fume hood).

g. Place ampicillin discs in a petri plate. Also place sterile BBL discs in another petri plate and add 10 ul of the crystal violet stock (1mg/ml) solution.

h. Just prior to the actual test time, thaw the required amount of S9 and prepare the + S9 mix (see Table 4 and Appendix A).

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i. Set up the assay station with cultures, +S9 mix, and -S9 mix in an ice bath with lead donuts holding the flasks in place and sterile repipets for dispensing.

E. Conducting the Assay

The conduct of the assay has been streamlined into an assembly line process requiring two laboratory personnel. Dispensing test samples, agar, cells and ± S9 mix is performed by one person, and the plating by the second experimenter.

1. Titers: Plate 0.1 ml of the 10^{-6} titer dilution with 0.1 ml of the Super His solution (50.42 ug/ml L-histidine HCL) and 2.5 ml soft agar supplemented with 0.05 mM biotin and 0.05 mM histidine HCl on agar plates with and without ampicillin. Plate in triplicate.

2. Plate samples: Plate O&C (--S9, -S9 and +S9) first, then the first DMSO (or other solvent) control (±S9), and followed by the appropriate positive controls for each culture [See Appendix A]. Follow with the random order of test samples with more DMSO (or other solvent) controls interspersed throughout the rest of the experiment (depending on the experimental size) and at the end.

   a. The order of addition to sterile test tubes is important **[4,37]**. The following order has been adopted in order to minimize solvent toxicity to the cells, to prevent accidental contamination of the cultures (or other dispenser tips) and to prevent possible thermal shock to the S9 enzyme systems.

      1. 0.1 ml of sample, solvent, or positive control.
      2. 2.5 ml of soft agar supplemented with 0.05 mM biotin and 0.05 mM L-hisitidine HCl.
      3. 0.1 ml of the overnight culture.
      4. 0.5 ml of either +S9 mix or -S9 mix.

   b. Mix well by vortexing.

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c. Pour mixture directly on Vogel Bonner minimal glucose agar plates. Tilt and swirl the plate to spread evenly. Allow to harden.

3. Markers: For the ampicillin disc and crystal violet marker checks, plate 0.1 ml of the overnight culture, 0.1 ml of Super His (50.42 ug/ml L-histidine HCl) and 2.5 ml soft agar supplemented with 0.05 mM biotin and 0.05 mM histidine HCl. Allow soft agar overlay to harden and then place discs with the appropriate chemical on the VBMG agar plates with sterile tweezers.

4. Sterility Checks: At the end of the experiment, all of the component solutions of the assay (See Table 3; 0.1 ml DMSO, 0.5 ml -S9 mix, 0.5 ml +S9 mix, 0.5 ml S9 fraction, 0.1 ml TA98 and/or TA100, 0.1 ml Super His solution, saline, and/or 0.1 ml deionized distilled water) should be plated in a soft agar overlay so that contamination can be traced to a source, should it occur. Each of these should be plated on a Vogel Bonner minimal glucose agar plate and on a nutrient agar plate.

F. Incubation

Invert plates after the soft agar has hardened and incubate at 37°C for 3 days (72 hours) in the dark. It has been recommended [4,14] that plates be placed within the incubator as soon as possible and certainly within an elapsed period of one hour. To achieve this, the assay has been modified in blocks of time so that three stacks of twelve plates each are placed in a 37°C incubator as soon as the soft agar has hardened. It is generaly recommended that the plates be counted immediately after the 3 day incubation period. If counting cannot be done immediately after this specified incubation period, the plates may be refrigerated briefly (no more than 2 days) before counting. Placement of plates in the incubator (location), and height of stacking has been shown to affect results in the assay [4, 69]. Because of this, uniform stacks of 12 plates are placed throughout the incubator. The random running order of samples and sample

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doses is thought to help minimize the effects of variation due to the location of plates in the incubator.

G. Counting and Data Analysis

Since hand counting is both laborious and time consuming, machine counting was instituted with a Biotran counter and an established hand to machine count correction curve [38]. When this instrument was replaced by the Artek [25], a new hand to machine count correction curve was determined [39]. Using the bar code labels to interface the counting process to the computer [12], plates are counted and plots are automatically generated. An example is shown in Figure 1. Note: Colonies on titer and toxicity assay [42] plates are counted manually.

Data can also be recorded and calculated manually using standard forms (Tables 5 and 6). The average solvent control (usually DMSO) is the background count normally subtracted for -S9 and +S9 plates. The specific activity (revertants/mg) is the slope calculated from the linear portion of a sample dose response curve. It is used to compare the activity of samples. Normally an analysis of variance is performed (using Minitab or RS/1) on the data to determine if the differences between the specific activities are significant. Plots can be generated manually using APL programs [36], Easy Graph or RS/1. APL plots are stored in designated notebooks for reference.

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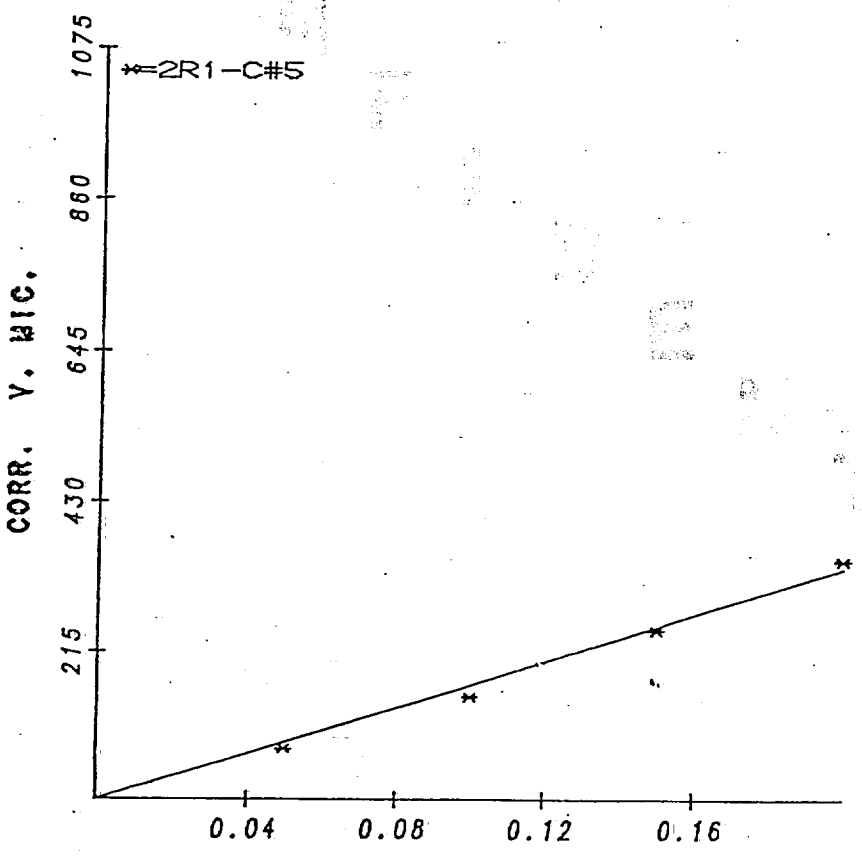
Figure 1. Sample APL Plot of 2R1 Data.

	DOSE	PLATE 4	PLATE 5	PLATE 6	CORR AV456	CORR. AV. MIC.
2R1-C#5	.0500	108	93	115	143.25	75.40
	.1000	180	165	141	220.32	152.47
	.1500	216	236	242	314.61	246.76
	.2000	279	306	330	414.80	346.95



8411/15 TA98 AR20 2R1-C#5
BG1= 36.33 BG2= 49.89
CORR. BACKGNDS 49.41 67.85
SLOPE OF LINE 1672.30

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Table 5. Salmonella/Microsome Assay: Control Data Sheet

N.B./Exp.#(or page)/Date _____/_____/__ - __ - _____ Enzyme: Batch#_____,ml of S9/ml of mix_____

Sterility Checks:	Vogel Bonner Plate	Nutrient Agar Plate
Overlay (2.5ml "soft agar")		
Overlay + DMSO (100ul)		
Overlay + "-S9 Mix" (0.5ml)		
Overlay + "+S9 Mix" (0.5ml)		
Overlay + "S9 Fraction" (0.5ml)		
Overlay + TA98 Cells (0.1ml)		
Overlay + TA100 Cells (0.1ml)		
Overlay + Super His (0.1ml)		
Overlay + "H_2O" (0.1ml)		

Machine Settings:
Size_____
Area_____
Sens_____

Klett Used:
TA98_____
TA100_____

Duration of Exp._____

Salmonella Strain and Control Data		-S9 Mix Plate No. 1	2	3	Avg.	Corr Avg.	Corr Avg. -BG	+S9 Mix Plate No. 4	5	6	Avg.	Corr Avg.	Corr Avg. -BG
TA98	Overlay + Cells	+(-S9)											
		-S9											
	DMSO-1												
	DMSO-2												
	DMSO-3												
	DMSO-4 (50 / 100 µl)												
	DMSO-5												
	DMSO-6												
	DMSO-7												
	2AA (.5 µg/plate/100µl)												
	2NF (2.5µg/plate/100µl)												
	2R1 CSC .05mg/plate/100µl												
	B# .10												
	.15												
	.20												
TA100	Overlay + Cells	+(-S9)											
		-S9											
	DMSO-1												
	DMSO-2												
	DMSO-3												
	DMSO-4 (50 / 100 µl)												
	DMSO-5												
	DMSO-6												
	DMSO-7												
	2AA (.5 µg/plate/100µl)												
	NaN_3 (.5µg/plate/100µl)												
	2R1 CSC .05mg/plate/100µl												
	B# .10												
	.15												
	.20												

TA98 Stock_____

Titers:	NA^{-amp}	NA^{+amp}
(6)		
(6)		
(6)		
Avg		

Response to:
Crystal violet_____
Ampicillin_____
2R1 CSC_____rev/mg

Klett Readings:
#1_____
#2_____
#3_____

TA100 Stock_____

Titers:	NA^{-amp}	NA^{+amp}
(6)		
(6)		
(6)		
Avg		

Response to:
Crystal violet_____
Ampicillin_____
2R1 CSC_____rev/mg

Klett Readings:
#1_____
#2_____
#3_____

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Table 6. Salmonella/Microsome Assay: Experimental Data Sheet

Fl Sec	Samples	+S9 Plates 4	5	6	Avg	Corr Avg	Corr Avg -BG	SLOPE	Fl Sec	Samples	+S9 Plates 4	5	6	Avg	Corr Avg	Corr Avg -BG	SLOPE

H. Monitoring Experimental Control Data

The TA98 and TA100 control data are located in a common account accessible by Project 6906 personnel. The first step is to logon to the system (PM700), and connect to the common account. The following outlines the protocols for entering and reviewing data:

Entering Data:

@aplsf

terminal...tty

)LOAD APL:ENTERS

)MAXCORE 300

CALL 101,1 (option to enter TA98 data)

CALL 101,2 (option to enter TA100 data)

code> notebook no/experiment no

code <carriage return>

1 code ready starting in column XXX

data > p

mon.day month.day (Date of the experiment)

year> year (last two digits)

group> return

O+C (0.1 ml) only > average overlay + cells (--)

O+C (0.1 ml) -S9 > average overlay + cells (-S9)

O+C (0.1 ml) +S9 > average overlay + cells (+S9)

DMSO (0.1 ml) > return

DMSO (0.1 ml) -S9 > average DMSO -S9

DMSO (0.1 ml) +S9 > average DMSO +S9

2AA (0.3 ug) + BKG > return

2AA (0.3 ug) - BKG > return

2AA (0.5 ug) + BKG > average 2AA

2AA (0.5 ug) - BKG > average 2AA minus DMSO (+S9) average value

2NF (2.5 ug) + BKG > average 2NF

2NF (2.5 ug) - BKG > average 2NF minus DMSO (-S9) average value

2R1 (Slope/10) > slope divided by 10

Titer (-Amp) no dilution > average titer (VBMG - ampicillin plate)

1:5 > return

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	1:10	>	return
Titer (+Amp)	no dilution	>	average titer (VBMG + ampicillin plate)
Klett	no dilution	>	average Klett reading ——|
	1:5	>	return |--> **Optional entry**
	1:10	>	return ___|
O.D.	no dilution	>	O.D. average reading
	1:5	>	return
	1:10	>	return
Cryst. Violet (mm)		>	Crystal violet zone measurement
uv Sensitivity		>	return
Water		>	average control (for NaN_3 in TA100 -S9)

Data >return

store 101,1 (TA98)

store 101,2 (TA100)

Inform > return

Title wanted > return

)MON

@logout

Reviewing the Data:

@aplsf

terminal.. tty

)LOAD APL:TABLES

)MAXCORE 300

CALL 101,1 (TA98)

CALL 101,2 (TA100)

TABLE ALL (to see all of the data)

TABLE XXX (to see a specific set of data)

PAGE 0 ... return

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I. Toxicity Assay

One of the limitations of the standard plate incorporation assay is the inability to determine the toxicity of test compounds. A study was conducted to evaluate two toxicity tests [42]. The reader is referred to

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this reference for the adopted recommendations and protocol for toxicity testing using the S/M tester strains. Note: This toxicity assay is used only when testing potential tobacco ingredients and other unknown samples. It is not used in the routine testing of CSCs.

VI. ACKNOWLEDGMENTS

All along, the development and use of the S/M assay has been a team effort. This report has taken a long time to evolve into the finished product presented here. This could not have happened without the help of L. H. Thompson, Colleen Deubler and Sarah Coleman. The author would like to recognize their support, comments, suggestions, general editing and contributions to this report and to the further development of the S/M assay. To all of the S/M personnel, past and present, I would like to express my deep gratitude to have had the priviledge to document their collective achievements.

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APPENDIX A. PROCEDURES FOR MAKING SOLUTIONS, CULTURE MEDIA, PLATE AGAR MEDIA, CSC OR POSITIVE CONTROL STOCK SOLUTIONS AND WORKING DILUTIONS, ETC.

1. Ampicillin Solutions:

A. For Plate Incorporation (Culture Reisolation):

1. Make up a **14.53 mg/ml ampicillin trihydrate solution:**
   a. Weigh out 0.07265 g of ampicillin trihydrate (FW = 403.47; anhydrous FW = 349.4).
   b. Dilute to 5 ml volume with sterile 0.02 N NaOH.
   c. Mix well to dissolve. Store frozen at -20°C.
2. Add 2 ml of the 14.53 mg/ml ampicillin trihydrate solution to one liter of ~45°C sterile agar [either Vogel Bonner minimal glucose agar media (solution 12.B) or Oxoid nutrient agar media (solution 9.B)] to give a final concentration of ~ **25 ug/ml** of ampicillin in the agar media. Dispense 30 ml of the agar media per plate.

B. For incorporation in Oxoid nutrient broth culture media (Culture Reisolation):

1. Make up a **1.479 mg/ml ampicillin trihydrate solution:**
   a. Weigh out 0.01479 g of ampicillin trihydrate (FW = 403.468; anhydrous FW = 349.4).
   b. Dilute to 10 ml volume with sterile 0.02 N NaOH.
   c. Mix well to dissolve.
2. Add 2 ml of the 1.479 mg/ml ampicillin solution to 100 ml of sterile Oxoid nutrient broth (solution 8.B) at room temperature to give a final concentration of ~ **25 ug/ml** ampicillin in the broth media.

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2. Biotin Solutions:

A. For soft (top) agar supplement to test for the histidine requirement:

1. Make up a **0.5 mM biotin** solution:

a. Weigh out 0.12215 g of D-biotin (FW = 244.3) into one liter of deionized distilled water.

b. Mix well to dissolve.

c. Filter sterilize through a 0.2 u Nalgene filter unit and store refrigerated.

d. Add 10 ml to 100 ml of soft (top) agar.

e. Use 2.5 ml of this biotin supplemented soft agar (solution 10) to plate 0.1 ml of a test culture. Incubate the plate overnight at 37°C. There should be no bacterial growth evident since there is a histidine requirement for the normal growth of these strains.

B. For Routine Soft (Top) Agar Supplementation:

1. Make up a **0.5 mM biotin + 0.5 mM L-histidine HCl solution:**

a. Weigh out D-biotin and L-histidine HCl and QS to the appropriate volume with deionized distilled water:

	g/100 ml	g/200 ml	g/500 ml	g/liter
L-histidine HCl (FW = 191.7)	0.009585 g	0.01917 g	0.047925 g	0.09585 g
D-biotin (FW = 244.3)	0.012215 g	0.02443 g	0.061075 g	0.12215 g

b. Mix well to dissolve. At room temperature, it may take all day to dissolve. Recent recommendations to heat the water to the boiling point have been made by Maron and Ames **[14]**.

c. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

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2. Add 10 ml of this solution to each 100 ml of sterile soft agar (solution 10). Use 2.5 ml of this supplemented soft

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(top) agar solution for plating the test compound, the culture and the S9 mix during routine testing.

C. OPTIONAL METHOD--For Soft (Agar) Supplement used to Plate Viable Counts (Titers) on Vogel Bonner Minimal Glucose Agar Plates:

1. Make up **0.5 mM biotin + 0.66 ug L-histidine HCl/ml** solution:

a. Weigh out D-biotin and L-histidine HCl and QS to the appropriate volume with deionized distilled water:

	g/100 ml	g/200 ml	g/500 ml	g/liter
L-histidine HCl (FW = 191.7)	0.06600 g	0.13200 g	0.33000 g	0.6600 g
D-biotin (FW = 244.3)	0.012215 g	0.02443 g	0.061075 g	0.12215 g

b. Mix well to dissolve.

c. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

2. Add 10 ml of this solution to each 100 ml of sterile soft agar. Use 2.5 ml of this supplemented soft (top) agar solution for plating titer dilutions of each overnight (16 hour) culture on Vogel Bonner minimal glucose agar plates. [Note: This is an alternative or optional procedure for plating titers. Normally, 0.1 ml of a 50.42 ug/ml histidine solution (solution 3) is pipetted into a tube containing 0.1 ml of the titer dilution which is then plated using the routine soft agar (solution 10) supplemented with histidine and biotin (see solution 2.B.).]

3. Histidine Solutions:

A. Super His Solution - For Plating Viable Counts (Titers) on Vogel Bonner Minimal Glucose Agar Plates in conjunction with the Routine Soft (Top) Agar:

1. Make up a **50.42 ug/ml histidine HCl** solution:

a. Weigh out 0.05042 g of L-histidine HCl.

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b. QS to 100 ml with deionized distilled water.

c. Mix well to dissolve.

d. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

2. Add 0.1 ml to each sterile culture tube to be used for plating viable counts (titers), crystal violet sensitivity, ampicillin resistance and colony morphology. Next, add 0.1 ml of the appropriate titer dilution. Using 2.5 ml of the soft agar (supplemented with solution 2.B.), plate mixture on Vogel Bonner minimal glucose agar plates.

4. Crystal Violet Solution (1 mg/ml):

A. Weigh out 10.6 mg of crystal violet (94% purity).

B. QS to 10 ml volume with sterile deionized distilled water.

C. Mix well to dissolve.

D. To check the rfa (deep rough mutation) marker:

1. Add 10 ul of the 1 mg/ml crystal violet solution to a sterile 6.35 mm (1/4") diameter BBL blank sensi disc and then place on Vogel Bonner minimal glucose agar plate to which biotin and excess histidine have been added with 0.1 ml of the overnight (16 hour) cell culture.

2. Incubate at 37°C.

3. A zone of ~14-18 mm inhibition around each disc indicates that the rfa marker is intact.

5. Glucose Solution (20 g/100 ml) for Vogel Bonner Minimal Glucose Agar Plates:

A. Weigh out 200 g of dextrose.

B. QS to one liter volume with deionized distilled water.

C. Mix well to dissolve all of the dextrose (low heat may have to be used).

D. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

E. Using disposable sterile 100 ml graduated cylinders (or sterile 100 ml glass cylinder), add 100 ml of this glucose solution to 400

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ml of sterile 2X Vogel Bonner salts. This solution may be stored at room temperature under sterile conditions.

6. 0.9% Sodium Chloride (NaCl) Solution (Saline solution for making titer dilutions):

A. Weigh out 9 g NaCl.

B. QS to one liter volume with deionized distilled water.

C. Mix well to dissolve.

D. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

E. Use 4.5 ml of this sterile saline solution for making titer dilutions.

7. 0.02 N Sodium Hydroxide (NaOH) Solution (for making ampicillin solutions):

A. Weigh out 0.8 g NaOH and dilute to one liter volume with deionized distilled water.

B. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

C. Use to make up ampicillin solutions (See solutions 1.A. and 1.B.).

8. Oxoid Nutrient Broth Culture Media:

A. Oxoid nutrient broth fortified with 10 ug/ml L-histidine HCl:

1. Weigh out Oxoid Nutrient Broth No. 2 and L-histidine HCl:

	per 100 ml	per 500 ml	per 1 L	per 2 L
Oxoid nutrient broth No. 2	2.5 g	12.5 g	25 g	50 g
L-histidine HCl	1 mg	5 mg	10 mg	20 mg

2. QS to the appropriate volume with deionized distilled water.

3. Mix well to dissolve.

4. Divide this mixture into 100 ml aliquots in 250 ml screw capped Erlenmeyer flasks.

5. Autoclave at 250° F for 30 minutes and store at room temperature.

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6. Use to dispense the appropriate volumes for overnight (16 hour) cultures, dilutions of overnight cultures or for blanks used to measure the optical density of overnight (16 hour) cultures on a Klett-Summerson photoelectric colorimeter or the Hitachi spectrophotometer.

B. Oxoid nutrient broth fortified with 10 ug/ml of L-histidine HCl and 25 ug/ml ampicillin (Culture reisolation):

1. Weigh out Oxoid nutrient broth No. 2 and L-histidine HCl (see chart for solution 8.A. above).
2. QS to the appropriate volume with deionized distilled water.
3. Mix well to dissolve.
4. Divide into 100 ml aliquots in 250 ml screw cap Erlenmeyer flasks.
5. Autoclave at 250° F for 30 minutes and cool media to room temperature.
6. Add 2 ml of a 1.479 mg/ml ampicillin trihydrate solution (see solution 1.B.) to 100 ml of culture media to give a final concentration of ~ 25 ug/ml of ampicillin.
7. Store a room temperature.
8. Use for growing up overnight cultures under ampicillin pressure.

9. Oxoid Nutrient Agar Plates:

A. Oxoid nutrient agar plates fortified with 10 ug/ml of L-histidine HCl:

1. Weigh out 25 g Oxoid nutrient broth No. 2, 10 mg L-histidine HCl, and 15 g Difco Bacto-agar into one liter of deionized distilled water (use 2 L Erlenmeyer flasks).
2. Autoclave for 45 minutes at 250 ° F.
3. Dispense 30 ml of the sterile nutrient agar media per plate using a New Brunswick Scientific dispenser pump and sterile tubing. [**Note**: Work performed in a laminar flow hood will minimize contamination of the plates. Also, plates should be inverted after they have hardened to prevent excess droplets

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of moisture from forming directly on the surface of the agar. Plates may be store refrigerated for several weeks.]

B. Oxoid nutrient agar plates fortified with 10 ug/ml of L-histidine HCl and 25 ug/ml of ampicillin (Culture reisolation):

1. Weigh out 25 g Oxoid nutrient broth No. 2, 10 mg L-histidine HCl and 15 g Difco Bacto-agar into one liter of deionized distilled water.
2. Autoclave for 45 minutes at 250° F.
3. Cool to approximately 45° C in a water bath.
4. Add 2 ml of a 14.53 mg/ml ampicillin trihydrate solution (see solution 1.A.) to give approximately 25 ug of ampicillin per ml of agar media.
5. Dispense 30 ml of the sterile agar media per plate using a New Brunswick Scientific dispenser pump. **[See previous note.]**

10. Soft (Top) Agar for Overlays:

A. Weigh out Difco Bacto-agar and NaCl into Erlenmeyer flasks:

	per 100 ml	per 500 ml	per 1 L
Difco Bacto-agar	0.6 g	3.0 g	6.0 g
NaCl	0.5 g	2.5 g	5.0 g
deionized distilled water	100 ml	500 ml	1000 ml
--after autoclaving			
0.5 mM histidine + 0.5 mM biotin (see 2.B.)	10 ml	50 ml	100 ml
Autoclave time	30 minutes	40 minutes	45 minutes

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B. Add the appropriate volume of deionized distilled water.

C. Autoclave for the appropriate time at 250°F.

D. Cool media to ~ 45°C in a heated water bath.

E. Add 10 ml of the appropriate soft agar supplement (histidine-biotin solutions) per 100 ml of soft agar before dispensing 2.5 ml of the supplemented soft agar into the culture tubes used for plating.

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11. Vogel Bonner 50X salt solution for making Vogel Bonner Minimal Glucose agar plates [Reference 43]:

A. Weigh out the components for this solution separately:

	per 1 L	per 2 L	per 3 L	per 4 L	per 16 L
ml deionized distilled water	670 ml	1340 ml	2010 ml	2680 ml	10,720 ml
g magnesium sulfate, 7-hydrate	10 g	20 g	30 g	40 g	160 g
g citric acid, monohydrate	100 g	200 g	300 g	400 g	1600 g
g potassium phosphate, dibasic, anhydrous	500 g	1000 g	1500 g	2000 g	8000 g
g sodium ammonium phosphate, 4-hydrate	175 g	350 g	525 g	700 g	2800 g

B. Mix each compound completely in the order indicated with the specified volume of deionized distilled water in order to avoid precipitation. Adjust final volume if necessary with deionized distilled water.

C. Store at room temperature in a Nalgene dispenser (Note: a few ml of chloroform may be added as a preservative).

12. Vogel Bonner Minimal Glucose Agar:

A. For Test Plates:

1. Weigh out 15 g of Difco Bacto-agar into a one liter Erlenmeyer flask; add 500 ml deionized distilled water; autoclave for 40 minutes at 250° F.
2. Measure 20 ml of the Vogel Bonner 50 X salt solution (see solution 11) into a one liter Erlenmeyer flask; add 380 ml deionized distilled water; autoclave for 40 minutes at 250°F. Note: This component solution can be made up, sterilized and stored at room temperature ahead of time.
3. Add 100 ml sterile glucose solution to the salt portion (2X sterile Vogel Bonner salts).

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4. Pour the salt/glucose solution into the agar solution.
5. Dispense 30 ml of the sterile agar media plus glucose per plate using a New Brunswick Scientific dispenser pump and sterile tubing. [**Note**: Work performed in a laminar flow hood will minimize contaminants on the plates. Plates should be inverted after they have hardened in order to prevent the formation of moisture droplets on the surface of the agar. The plates may be stored refrigerated for several weeks.]

B. For Vogel Bonner plates that could be used to plate viable counts to test for ampicillin resistance in strains TA98 and TA100:

1. Prepare Vogel Bonner minimal glucose agar media as above (see solution 12.A.).
2. Before dispensing, add 2 ml of a 14.53 mg/ml ampicillin trihydrate solution (see solution 1.A.) per liter of sterile agar to give a final concentration of ~ 25 ug/ml of ampicillin in the agar media.
3. Dispense 30 ml of the sterile agar/glucose/ampicillin media per plate using a New Brunswick Scientific dispenser pump and sterile tubing. **[See previous note above.]**

13. Sodium Phosphate Buffer:

A. Stock Solutions: Make up stock solutions according to the following table. Solution A is the sodium phosphate, monobasic solution. Solution B is the sodium phosphate, dibasic solution.

Stock Solution	Compound	g/500 ml	g/1 liter
0.2 M NaH_2PO_4 (soln A)	$NaH_2PO_4(H_2O)$	13.6 g	27.2 g
0.2 M Na_2HPO_4 (soln B)	$Na_2HPO_4(7H_2O)$	26.83 g	53.66 g

1. Mix up these two stock solutions separately.
2. QS to the appropriate volume with deionized distilled water.

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B. Sodium Phosphate Buffer, pH = 7.4: Make up buffer according to the following table.

Stock Solution	ml/100 ml	ml/500 ml	ml/1 liter	ml/2 liter
(A) 0.2 M NaH_2PO_4	19 ml	95 ml	190 ml	380 ml
(B) 0.2 M Na_2HPO_4	81 ml	405 ml	810 ml	1620 ml
Autoclave time	30 minutes	40 minutes	45 minutes	55 minutes

1. Check the pH of the final solution and adjust to pH 7.4 if necessary with the stock solutions [the NaH_2PO_4 solution (solution A) will lower the pH; the Na_2HPO_4 solution (solution B) will raise the pH].
2. Autoclave at 250 °F for the appropriate time.

14. Ames Salt Solution for the S9 Mix (0.4 M $MgCl_2$ + 1.65 M KCl):

A. Make up the salt solution according to the following table:

Compound	g/100 ml	g/ 500 ml	g/1 liter
$MgCl_2$	8.13 g	40.65 g	81.3 g
KCl	12.3 g	61.5 g	123.3 g
Autoclave time	30 min.	40 min.	45 min.

B. QS to the appropriate volume with deionized distilled water.

C. Mix well to dissolve.

D. Autoclave at 250°F for the appropriate time.

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15. -S9 Mix:

A. Make up the -S9 solution according to the following table:

Solution	per 1 ml	per 4 L
Ames salt solution (solution 14)	0.02 ml	80 ml
Sodium phosphate buffer (" 13.B)	0.50 ml	2000 ml
deionized distilled water	0.41 ml	1640 ml

Note: the sodium phosphate buffer and Ames salt solutions must be completely cooled before mixing, otherwise, a precipitate will occur.

B. Mix solutions and water thoroughly.

C. Filter sterilize through 0.2 u Nalgene filter units (Note: Do not autoclave this final solution since a precipitate will form) and store refrigerated.

16. + S9 Mix Solutions:

A. FOR STANDARD PLATE INCORPORATION ASSAY - + S9 Mix [8 mM Magnesium chloride; 33 mM potassium chloride; 5 mM glucose-6-phosphate; 4 mM NADP (TPN); 100 mM sodium phosphate buffer, pH 7.4; 0.070 ml of S9/ml mix (35 ul of S9 per plate)]:

Prepare the +S9 mix solution fresh the day of the experiment and keep on ice according to the experimental volume needed on the following chart [Note: the appropriate amount of the -S9 mix (solution 15 above) can be used for this recipe instead of the individual salt, buffer and water solutions.]:

Volume ======>	1 ml	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml
ml -S9 Mix	0.93	4.65	9.3	13.95	18.6	23.25	27.9
ml S9	0.07	0.35	0.7	1.05	1.4	1.75	2.2
g NADP	0.00343	0.01717	0.03434	0.05152	0.06869	0.08586	0.10303
g G-6-P	0.00146	0.00730	0.01460	0.02190	0.02920	0.03650	0.04380

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[+S9 Mix for Standard Plate Incorporation Assay (35 ul S9/plate) continued]

Volume ======>	35 ml	40 ml	45 ml	50 ml	55 ml	60 ml	65 ml
ml -S9 Mix	32.55	37.2	41.85	46.5	51.15	55.8	60.45
ml S9	2.45	2.8	3.15	3.5	3.85	4.2	4.55
g NADP	0.12020	0.13738	0.15455	0.17172	0.18889	0.20606	0.22324
g G-6-P	0.05110	0.05840	0.06570	0.07300	0.08030	0.08760	0.09490

Volume ======>	70 ml	75 ml	80 ml	85 ml	90 ml	95 ml	100 ml
ml -S9 Mix	65.1	69.75	74.4	79.05	83.7	88.35	93.0
ml S9	4.9	5.25	5.6	5.95	6.3	6.65	7.0
g NADP	0.24041	0.25758	0.27475	0.29192	0.30910	0.32627	0.34344
g G-6-P	0.10220	0.10959	0.11680	0.12410	0.13140	0.13870	0.14600

Volume ======>	105 ml	110 ml	115 ml	120 ml	125 ml	130 ml	135 ml
ml -S9 Mix	97.65	102.3	106.95	111.6	116.25	120.9	125.55
ml S9	7.35	7.7	8.05	8.4	8.75	9.1	9.45
g NADP	0.36061	0.37778	0.39496	0.41213	0.42930	0.44647	0.46364
g G-6-P	0.15330	0.16060	0.16790	0.17520	0.18250	0.18980	0.19710

Volume ======>	140 ml	145 ml	150 ml	155 ml	160 ml	165 ml	170 ml
ml -S9 Mix	130.2	134.85	139.5	144.15	148.8	153.45	158.1
ml S9	9.8	10.15	10.5	10.85	11.2	11.55	11.9
g NADP	0.48082	0.49799	0.51516	0.53233	0.54950	0.56668	0.58385
g G-6-P	0.20440	0.21170	0.21900	0.22630	0.23360	0.24090	0.24820

Volume ======>	175 ml	180 ml	185 ml	190 ml	195 ml	200 ml	205 ml
ml -S9 Mix	162.75	167.4	172.05	176.7	181.35	186.0	190.65
ml S9	12.25	12.6	12.95	13.3	13.65	14.0	14.35
g NADP	0.60102	0.61819	0.63536	0.65254	0.66971	0.68688	0.70405
g G-6-P	0.25550	0.26280	0.27010	0.27740	0.28470	0.29200	0.29930

Volume ======>	210 ml	215 ml	220 ml	225 ml	230 ml	235 ml	240 ml
ml -S9 Mix	195.3	199.95	204.6	209.25	213.9	218.55	223.2
ml S9	14.7	15.05	15.4	15.75	16.1	16.45	16.8
g NADP	0.72122	0.73840	0.75557	0.77274	0.78991	0.80708	0.82426
g G-6-P	0.30660	0.31390	0.32120	0.32850	0.33580	0.34310	0.35040

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[+S9 Mix for Standard Plate Incorporation Assay (35 ul S9/plate) continued]

Volume ======>	245 ml	250 ml	255 ml	260 ml	265 ml	270 ml	275 ml
ml -S9 Mix	227.85	232.5	237.15	241.8	246.45	251.1	255.75
ml S9	17.15	17.5	17.85	18.2	18.55	18.9	19.25
g NADP	0.84143	0.85860	0.87577	0.89294	0.91012	0.92729	0.94446
g G-6-P	0.35770	0.36500	0.37230	0.37960	0.38690	0.39420	0.40150

Volume ======>	280 ml	285 ml	290 ml	295 ml	300 ml	305 ml	310 ml
ml -S9 Mix	260.4	265.05	269.7	274.35	279.0	283.65	288.3
ml S9	19.6	19.95	20.3	20.65	21.0	21.35	21.7
g NADP	0.96163	0.97880	0.99598	1.01315	1.03032	1.04749	1.06466
g G-6-P	0.40880	0.41610	0.42340	0.43070	0.43800	0.44530	0.45260

Volume ======>	315 ml	320 ml	325 ml	330 ml	335 ml	340 ml	345 ml
ml -S9 Mix	292.95	297.6	302.25	306.9	311.55	316.2	320.85
ml S9	22.05	22.4	22.75	23.1	23.45	23.8	24.15
g NADP	1.09184	1.09901	1.11618	1.13335	1.15052	1.16770	1.18487
g G-6-P	0.45990	0.46720	0.47450	0.48180	0.48910	0.49640	0.50370

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B. FOR S9 COMPARISONS ONLY - + S9 Mix [8 mM Magnesium chloride; 33 mM potassium chloride; 5 mM glucose-6-phosphate; 4 mM NADP (TPN); 100 mM sodium phosphate buffer, pH 7.4; 0.090 ml of S9/ml mix (45 ul of S9 per plate)]:

Prepare the +S9 mix solution fresh the day of the experiment and keep refrigerated or on ice according to the experimental volume needed on the following chart:

Volume ======>	1 ml	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml
ml -S9 Mix	0.91	4.55	9.1	13.65	18.2	22.75	27.3
ml S9	0.09	0.45	0.9	1.35	1.8	2.25	2.7
g NADP	0.00343	0.01717	0.03434	0.05152	0.06869	0.08586	0.10303
g G-6-P	0.00146	0.00730	0.01460	0.02190	0.02920	0.03650	0.04380

Volume ======>	35 ml	40 ml	45 ml	50 ml	55 ml	60 ml	65 ml
ml -S9 Mix	31.85	36.4	40.95	45.5	50.05	54.6	59.15
ml S9	3.15	3.6	4.05	4.5	4.95	5.4	5.85
g NADP	0.12020	0.13738	0.15455	0.17172	0.18889	0.20606	0.22324
g G-6-P	0.05110	0.05840	0.06570	0.07300	0.08030	0.08760	0.09490

Volume ======>	70 ml	75 ml	80 ml	85 ml	90 ml	95 ml	100 ml
ml -S9 Mix	63.7	68.25	72.8	77.35	81.9	86.45	91.0
ml S9	6.3	6.75	7.2	7.65	8.1	8.55	9.0
g NADP	0.24041	0.25758	0.27475	0.29192	0.30910	0.32627	0.34344
g G-6-P	0.10220	0.10959	0.11680	0.12410	0.13140	0.13870	0.14600

Volume ======>	105 ml	110 ml	115 ml	120 ml	125 ml	130 ml	135 ml
ml -S9 Mix	95.55	100.1	104.65	109.2	113.75	118.3	122.85
ml S9	9.45	9.9	10.35	10.8	11.25	11.7	12.15
g NADP	0.36061	0.37778	0.39496	0.41213	0.42930	0.44647	0.46364
g G-6-P	0.15330	0.16060	0.16790	0.17520	0.18250	0.18980	0.19710

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[+S9 Mix for S9 comparisons (45 ul S9/plate) continued]

Volume ======>	140 ml	145 ml	150 ml	155 ml	160 ml	165 ml	170 ml
ml -S9 Mix	127.4	131.95	136.5	141.05	145.6	150.15	154.7
ml S9	12.6	13.05	13.5	13.95	14.4	14.85	15.3
g NADP	0.48082	0.49799	0.51516	0.53233	0.54950	0.56668	0.58385
g G-6-P	0.20440	0.21170	0.21900	0.22630	0.23360	0.24090	0.24820

Volume ======>	175 ml	180 ml	185 ml	190 ml	195 ml	200 ml	205 ml
ml -S9 Mix	159.25	163.8	168.35	172.9	177.45	182.0	186.55
ml S9	15.75	16.2	16.65	17.1	17.55	18.0	18.45
g NADP	0.60102	0.61819	0.63536	0.65254	0.66971	0.68688	0.70405
g G-6-P	0.25550	0.26280	0.27010	0.27740	0.28470	0.29200	0.29930

Volume ======>	210 ml	215 ml	220 ml	225 ml	230 ml	235 ml	240 ml
ml -S9 Mix	191.1	195.65	200.2	204.75	209.3	213.85	218.4
ml S9	18.9	19.35	19.8	20.25	20.7	21.15	21.6
g NADP	0.72122	0.73840	0.75557	0.77274	0.78991	0.80708	0.82426
g G-6-P	0.30660	0.31390	0.32120	0.32850	0.33580	0.34310	0.35040

Volume ======>	245 ml	250 ml	255 ml	260 ml	265 ml	270 ml	275 ml
ml -S9 Mix	222.95	227.5	232.05	236.6	241.15	245.7	250.25
ml S9	22.05	22.5	22.95	23.4	23.85	24.3	24.75
g NADP	0.84143	0.85860	0.87577	0.89294	0.91012	0.92729	0.94446
g G-6-P	0.35770	0.36500	0.37230	0.37960	0.38690	0.39420	0.40150

Volume ======>	280 ml	285 ml	290 ml	295 ml	300 ml	305 ml	310 ml
ml -S9 Mix	254.8	259.35	263.9	268.45	273.0	277.55	282.1
ml S9	25.2	25.65	26.1	26.55	27.0	27.45	27.9
g NADP	0.96163	0.97880	0.99598	1.01315	1.03032	1.04749	1.06466
g G-6-P	0.40880	0.41610	0.42340	0.43070	0.43800	0.44530	0.45260

Volume ======>	315 ml	320 ml	325 ml	330 ml	335 ml	340 ml	345 ml
ml -S9 Mix	286.65	291.2	295.75	300.3	304.85	309.4	313.95
ml S9	28.35	28.8	29.25	29.7	30.15	30.6	31.05
g NADP	1.09184	1.09901	1.11618	1.13335	1.15052	1.16770	1.18487
g G-6-P	0.45990	0.46720	0.47450	0.48180	0.48910	0.49640	0.50370

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17. **Positive Controls**

Note: Positive control stock solutions are prepared in a glove box. Refer to reference 51 for the procedures. Test solution are prepared in a chemical fume hood.

A. 2AA (2-aminoanthracene):

1. Stock Solution:

a. Weigh out 40 mg of 2AA and dissolve in 20 ml DMSO to give a stock concentration of 2 mg/ml.

b. Dispense 0.1 ml aliquots of the 2 mg/ml 2AA solution into capped cryule tubes.

c. Store at -20°C. This makes approximately 200 tubes of the 2AA stock (200 ug/0.1 ml).

2. Testing Solution (0.5 ug/plate):

a. Add 1.9 ml DMSO to the 0.1 ml of thawed 2AA stock to make a 100 ug/ml 2AA solution.

b. Take 0.1 ml of the 100 ug/ml 2AA solution and dilute with 1.9 ml DMSO to make a 5 ug/ml 2AA solution.

c. As a positive control for strains TA98 and TA100 with metabolic activation, plate 0.1 ml of the 5 ug/ml 2AA solution in the standard assay (0.5 ug of 2AA/plate).

B. 2NF (2-nitrofluorene):

1. Stock Solution:

a. Weigh out 10 mg of 2NF and dissolve in 20 ml DMSO to give a stock concentration of 0.5 mg/ml.

b. Dispense 0.1 ml aliquots of the 0.5 mg/ml 2NF solution into capped cryule tubes.

c. Store at -20°C. This makes approximately 200 tubes of the 2NF stock (50 ug/0.1 ml).

2. Testing Solution (2.5 ug/plate):

a. Add 1.9 ml DMSO to the 0.1 ml of thawed 2NF stock to make a 25 ug/ml 2NF solution.

b. As a positive control for strain TA98 without metabolic activation, plate 0.1 ml of the 25 ug/ml 2NF solution in the standard assay (2.5 ug of 2NF/plate).

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C. Sodium Azide (NaN_3) :

1. Stock Solution:

a. Weigh out 40 mg of sodium azide and dissolve in 20 ml sterile deionized distilled water to give a stock concentration of 2 mg/ml.

b. Dispense 0.1 ml aliquots of the 2 mg/ml sodium azide solution into capped cryule tubes.

c. Store at room temperature. This makes approximately 200 tubes of the sodium azide stock (200 ug/0.1 ml).

2. Testing Solution (0.5 ug/plate):

a. Add 1.9 ml deionized distilled water to the 0.1 ml of sodium azide stock (stored at room temperature) to make a 100 ug/ml sodium azide solution.

b. Take 0.1 ml of the 100 ug/ml sodium azide solution and dilute with 1.9 ml deionized distilled water to make a 5 ug/ml sodium azide solution.

c. As a positive control for strain TA100 without metabolic activation, plate 0.1 ml of the 5 ug/ml sodium azide solution in the standard assay (0.5 ug of sodium azide per plate). The solvent control in this case is deionized distilled water.

18. Impaction trapped (IT) Cigarette Smoke Condensate (CSC) Solutions:

A. Preparation of IT CSC: See Reference 13 for IT CSC collection.

B. IT CSC Stock Solutions (10 mg/ml):

1. The weight of the CSC is provided by Project 6908 personnel.

2. Add DMSO directly to CSC in the impaction trap flask to give a stock concentration of 10 mg of CSC/ml in DMSO. Mix well to fully dissolve the CSC in the DMSO. These operations are routinely performed by Project 6908 personnel.

C. IT CSC Dilutions for Testing in the S/M assay: Routinely 0.1 ml of IT CSC dilutions are tested at concentrations of 0.5, 0.10, 0.15 and 0.20 mg of CSC per plate. These concentrations are normally in the linear range of the dose response curve. The following table gives the information for making these dilutions:

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[CSC]/0.1 ml/plate	ml of 10 mg/ml stock	ml DMSO
0.05 mg	0.050	0.950
0.10 mg	0.100	0.900
0.15 mg	0.150	0.850
0.20 mg	0.200	0.800

19. Test Compound (Potential Tobacco Ingredient) Solutions:

A. Obtain sample and obtain information on the concentration, solubility, solvent, etc.

B. Prepare dilutions of the sample in either DMSO or another appropriate solvent to give a log scale dose range of 0.0001, 0.001, 0.01, 0.1, 1.0, and 10.0 mg/plate. The actual dose scheme used may depend on the amount of the sample that is available for testing in the S/M assay. Normally, serial dilutions are made from a 100 mg/ml stock solution of each sample. The solvent control for each sample will depend on the solvent used.

20. UV Sensitivity Check: The purpose of this check is to confirm the uvrB mutation.

A. With a sterile wire loop, streak the overnight (16 hour) cultures on Oxoid nutrient agar plates.

B. Cover half of the petri plate with foil or other suitable material.

C. Irradiate the plate with germicidal shortwave (254 nm) uv light at a distance of 33 cm for 8 seconds.

D. Incubate at 37°C for 12-24 hours. The side of the petri plate exposed to the uv light should not show any viability.

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APPENDIX B. LIST OF CHEMICALS AND EQUIPMENT

Name of Chemical or Equipment	Supplier	Catalogue No.
2-aminoanthracene (2AA)	Sigma Chemical Company	A1381
Ampicillin, trihydrate (pfs)	Sigma Chemical Company	A6140
Ampicillin, PN 10 ug Oxoid single sensitive discs in cartridges	Fisher Scientific	B31264
Aroclor® 1254 induced microsomes (S9)	Litron Laboratories	
Artek Colony Counter	Artek System	Model 880
Autoclavable disposable bags	Fisher Scientific	(large) 01-815C (small) 01-815-1
Autoclave	American Sterilizer Co.	Eagle (2021 gravity)
Bioshields, 24" x 24" sterilization wrap sheets	American Scientific Products	A9350-124
D-biotin (pfs); FW = 244.3	Sigma Chemical Company	B4501
BBL blank sensi test discs (6.35 mm)	Fisher Scientific	B31039
Citric acid, monohydrate granular; Fisher Certified A.C.S.; FW = 210.14	Fisher Scientific	A104-500
Colony counter, Darkfield Quebec; American Optical Corporation	American Scientific	C8374-1
Crystal violet, Fisher certified stain	Fisher Scientific	C-581-100
Cryules, sterile Wheaton with screw caps	Wheaton Scientific	985731
Culture tubes, diSPo: 16 x 100 mm	American Scientific	T1285-6
Dextrose, Fisher Certified A.C.S.; D-glucose, anhydrous powder; FW = 180.16	Fisher Scientific	D-16-500
DMSO; dimethyl sulfoxide (pfs)	Sigma Chemical Company	D5879

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Difco Bacto-Agar	American Scientific Products		0140-01
diSPo plugs, 35-45 mm	American Scientific Products		T-1387
Eppendorf digital 100-1000 ul pipette with tip ejector	BioRad Labortories		22-33-360 2234 170-7
Erlenmeyer flasks, with screw caps	Fisher Scientific	250 ml 500 ml	10-093-B 10-093-C
D-glucose-6-phosphate, monosodium salt	Sigma Chemical Company		G7879
Graduated cylinder, 100 ml sterile, individually wrapped; polycarbonate	Fisher Scientific		8-558-10
Finnpipette, adjustable volume with automatic tip ejector	Cole Parmer		
A. 40-200 ul			J-6247-54
B. 200-1000 ul			J-6247-55
Finnpipettes, fixed volume with tip ejector:	Cole Parmer		
A. 10 ul			J-6247-11
B. 50 ul			J-6247-14
C. 100 ul			J-6247-15
D. 150 ul			J-6247-16
E. 200 ul			J-6247-17
F. 500 ul			J-6247-20
Harris -80°C freezer	Harris Manufacturing Company, Inc.		Model LTF-6-5-85
L-histidine HCl; monohydrochloride; monohydrate; Sigma grade; FW = 191.7	Sigma Chemical Company		H8125
Hitachi spectrophotometer	Hitachi		Model 150-20
Hot plate stirrer (Thermolyne)	VWR Scientific		33922156
Incubator, National	National Appliance Company		Model 5512
Klett-Summerson photoelectric colorimeter	VWR Scientific		22461-007
5 ml colorimeter tubes	VWR Scientific		22463-002
Labcon heavy duty utility cart	American Scientific Products		C-1252

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Source: https://www.industrydocuments.ucsf.edu/docs/gsgm0000

Laminar flow ultra clean work bench	Agnew Higgins, Inc.	
Loops, inoculating	American Scientific Products	N2010
Loop holder	American Scientific Products	N2000
Magnesium chloride $MgCl_2$ $6H_2O$	Fisher Scientific	M33-500
Magnesium sulfate, 7-hydrate crystal, Fisher Certified A.C.S.	Fisher Scientific	M63-500
Magnetic stirrers (Mono Mold)	Cole Parmer	small J-4770-20 medium J-4770-40 large J-4770-60
Mettler analytical balance, Model H51AR	Fisher Scientific	01-909-H51AR
Mettler balance, top loader, Model P1200	Fisher Scientific	1-913-106
Microscope (Wild) and power source	Bunton Instrument Company, Inc.	Model 20-90917
NADP, nicotinamide-adenine-dinucleotide phosphate, oxidized form, disodium salt (98%)	Boehringer Mannheim	240 354 (5 g)
Nalgene filter units, 0.2 u A. 115 ml B. 500 ml	Fisher Scientific	 120-0020 and 9-740 450-0020 and 9-740-25A
New Brunswick Scientific dispenser pump	New Brunswick Scientific	DP-100
2-nitrofluorene, 2NF	Aldrich Chemical Company	N1,675-4
Oxoid nutrient broth No. 2	Oxoid Canada Limited	CM67
Parafilm	American Scientific Products	P1150-4
Petri plates, 15 x 100 mm Kimble	American Scientific Products	D1906
Phenol Reagent solution 2N (Folin-Ciocalteau)	Fisher Scientific	SP24-100
Pipet-Aid Filler and	Fisher Scientific	13-681-15

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Dispenser			
Pipette tips, MLA (for Finnpipettes):	Fisher Scientific		
A. Small MLA; sterile; individually wrapped for 0-200 ul volumes			21-341-10
B. Large MLA; sterile; individually wrapped for 250-1000 ul volumes			21-343-10
Pipettes, sterile plugged; individually wrapped glass pipettes	American Scientific Products	1 ml 5 ml 10 ml	P4650-1Y P4650-5Y P4650-10Y
Potassium chloride	American Scientific		6858-500*NY
Potassium phosphate, dibasic; anhydrous powder; AR(ACS)	American Scientific Products		7092-500*NY
Potassium phosphate, monobasic	American Scientific Products		7100-500*NY
Salmonella typhimurium strains	Dr. Bruce N. Ames		
Sodium ammonium phosphate; Fisher certified ACS; FW = 209.07	Fisher Scientific		S-218-500
Sodium phosphate, monobasic crystal	Fisher Scientific		S-369-500
Sodium phosphate, dibasic, heptahydrate	Fisher Scientific		S-373-500
Sodium azide	Sigma Chemical Company		S-2002
Sodium chloride; Fisher certified ACS; crystal; FW = 58.44	Fisher Scientific		S-271-500
Sodium hydroxide, pellets	Fisher Scientific		S-318-500
Sterilization Tape: one inch	American Scientific		A9470-22
Test tube caps; disposable; autoclavable; size 16; Bacti capall (Kim Kap)	American Scientific		T1291-16
Test tube racks: For 16 x 100 mm tubes	Fisher Scientific		14-793-9 14-793-13
Universal repipette dispensers:	Lab Industries		

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[Appendix B continued...]

A. 0.5 ml dispenser		300 1/2A
B. 1.0 ml dispenser		3001A-U
C. 5.0 ml dispenser		3005A-U
Universal repipette plunger assembly:	Lab Industries	
A. 0.5 ml assembly		4020
B. 1.0 ml assembly		4022
C. 5.0 ml assembly		4026
Universal repipette indicator	Lab Industries	
A. 0.5 and 1.0 ml indicator		552-M-1
B. 5.0 ml indicator		552-M-5
uv light: closed end reflector with aluminum legs	American Ultra Violet Company	Model CE-10-CAL
lamp: germicidal shortwave (254 nm); cold cathode lamp (28 micro Watts per sq. cm at a distance of one meter)		Westinghouse Sterilamp 782L-10
Vial, 5 dram amber glass vials with screw caps	Owen, Minor and Bodecker	PW4005
Vial cap liners; teflon; for 5 dram vial caps; size 24	Arthur H. Thomas, Company	2390-H72
Vial rack; polycarbonate; for cryules	Arthur H. Thomas, Company	5151-S25
Vortexer, Thermolyne Maxi Mix	Fisher Scientific	12-814
Water bath, gyrotary shaker bath Lab Line Orbit Microprocessor shaker bath	Fisher Scientific	3545
platform		15-456-2M
250 ml flask clamps		14-272-4B
Water bath, Lauda circulating heating water bath; Model C3 T-1	American Scientific	W3243-1
Water bath, Precision holding bath	American Scientific Products	B7008-4
Zephiran chloride; 1:750 d aqueous solution	Owens, Minor and Bodecker	Z-493 HRI 8024-253402

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APPENDIX C. LIST OF SUPPLIERS

1. Agnew Higgins, Inc.
   7091 Belgrave Avenue
   Garden Grove, CA 92641

2. Aldrich Chemical Company
   940 West Saint Paul Avenue
   Milwaukee, Wisconsin 53233
   800-558-9160

3. Ames, Dr. Bruce N.
   Department of Biochemistry
   University of California, Berkeley
   Berkeley, CA 97418
   415-642-5163

4. American Scientific Products
   8855 McGraw Road
   Columbia, Maryland 21045
   800-638-2813

5. American Sterilizer Company
   701 Research Road
   Richmond, VA
   804-794-6871

6. American Ultra Violet Company
   450 Commerce Street
   Charthm, NJ
   201-635-8355

7. Artek Systems
   Farmindale, NY

8. Arthur H. Thomas, Co.
   Vine Street at Third
   P. O. Box 779
   Philadelphia, PA 19105
   215-574-4500

9. BioRad Laboratories
   P. O. Box 708
   220 Maple Avenue
   Rockville Centre, NY 11571
   516-764-2575

10. Boehringer Mannheim Biochemicals
    7941 Castleway Drive
    P.O. Box 50816
    Indianapolis, IN 46250
    800-428-5433

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11. Bunton Instrument Co., Inc.
615 South Stonestreet Avenue
Rockville, MD 20850
301-762-5515 or 5516

12. Central Virginia Medical Equipment Repair Company
12431 Donegal Road
Chesterfield, VA 23832
804-790-1088

13. Cole Parmer
7425 North Oak Park Avenue
Chicago, IL 60648
800-323-4340

14. Fisher Scientific
7633 Hull Street Road
Richmond, VA 23235
804-745-5535

15. General Scientific
8741 Landmark Road
P. O. Box 26509
Richmond, VA
804-264-7560

16. Harris Manufacturing, Co., Inc.
Treble Cove Industrial Park
Republic Road
No. Bellerica, MA 01862
617-667-5116

17. Hitachi, Ltd. Tokoyo, Japan
5-1, 1-chrome
Marunouchi, Chiyoda-Ku,
Tokoyo
800-421-2042

18. Lab Industries
180 22nd Street
Berkeley, CA 97418

19. Markson Science, Inc.
733 15th Street, NW
Suite 438
Washington, DC 20005
714-755-6655

20. National Appliance Co.
(subidiary of Heinckle Co.)
Portland, OR

21. New Brunswick Scientific
P. O. Box 986
44 Talmadge Road

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Edison, NJ 08817
201-287-1200

22. Owen, Minor and Bodecker
4825 Bethlehem Road
Richmond, VA
804-355-0681

23. Oxoid Canada, Ltd.
9017 Red Branch Road
Columbia, MD 21045

24. Sigma Chemical Company
P. O. Box 14508
St. Louis, MO 63178
800-325-3010

25. Thomas Scientific
99 High Hill Road and I-295
P.O. Box 99
Swedesboro, NJ 09095-0099
800-345-2100

26. VWR Scientific
6601 Amberton Drive
Baltimore, MD 21227
800-638-5722 or 301-796-8500

27. Wheaton Scientific
1000 North Tenth Street
Millville, NJ 08332
800-257-7047

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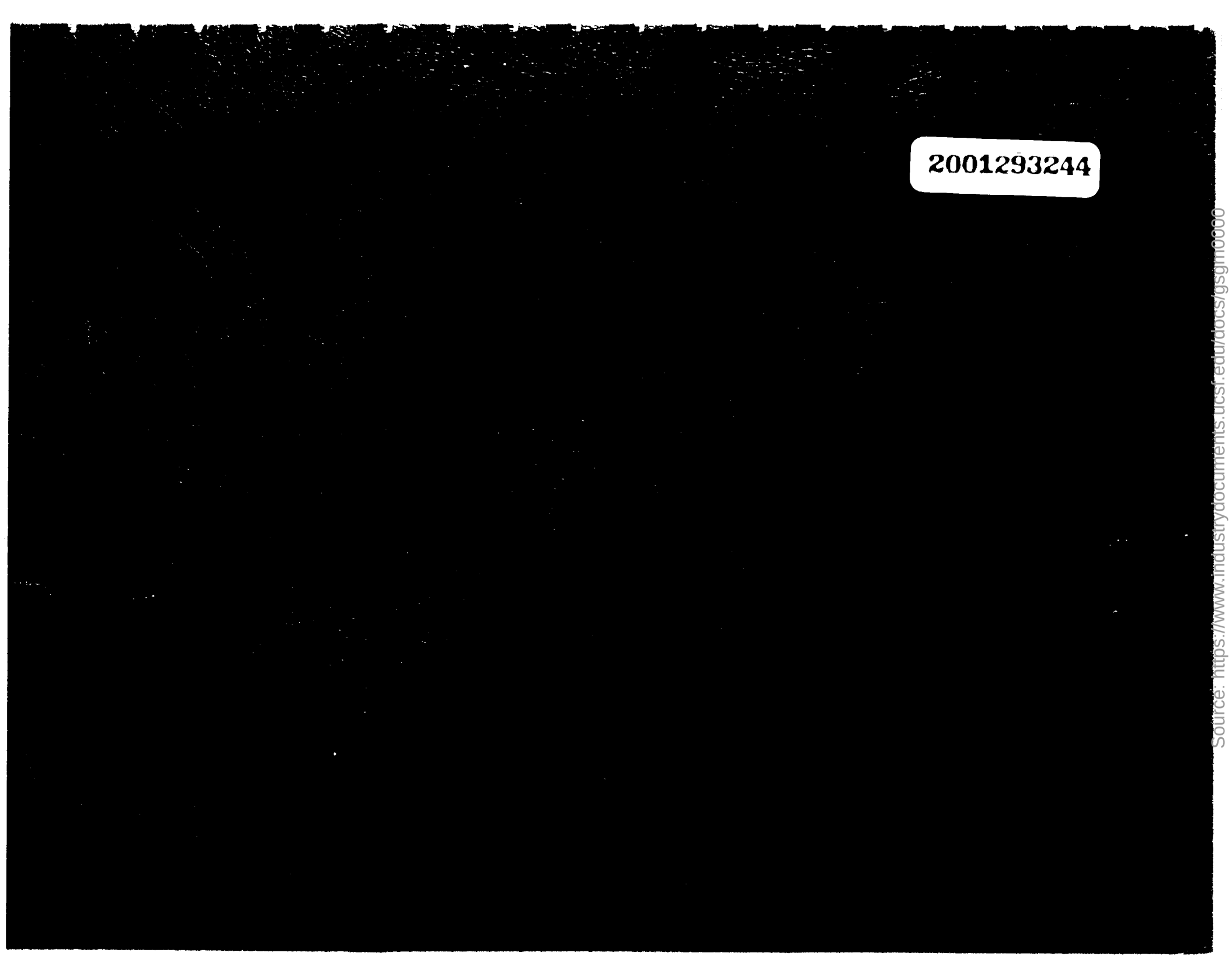
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